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## **Decreased renal accumulation of aminoglycoside reflects defective receptor-mediated endocytosis in cystic fibrosis and Dent's disease**

Raggi, C ; Fujiwara, K ; Leal, T ; Jouret, F ; Devuyst, O ; Terryn, S

**Abstract:** The clinical use of aminoglycoside (AG) antibiotics is limited by their renal toxicity, which is caused by drug accumulation in proximal tubule (PT) cells. Clinical studies reported that renal clearance of AG is enhanced in cystic fibrosis (CF) patients, which might reflect the role of CFTR in PT cell endocytosis. In order to assess the role of chloride transporters on the renal handling of AG, we investigated gentamicin uptake and renal accumulation in mice lacking functional CFTR (Cftr ( F/ F)) or knock-out for the Cl(-)/H(+) exchanger ClC-5 (Clcn5 ( Y/- )). The latter represent a paradigm of PT dysfunction and defective receptor-mediated endocytosis. As compared with controls, Cftr ( F/ F) and Clcn5 ( Y/- ) mice showed a 15% to 85% decrease in gentamicin accumulation in the kidney, respectively, in absence of renal failure. Studies on primary cultures of Cftr ( F/ F) and Clcn5 ( Y/- ) mouse PT cells confirmed the reduction in gentamicin uptake, although colocalization with endosomes and lysosomes was maintained. Quantification of endocytosis in PT cells revealed that gentamicin, similar to albumin, preferentially binds to megalin. The functional loss of ClC-5 or CFTR was reflected by a decrease of the endocytic uptake of gentamicin, with a more pronounced effect in cells lacking ClC-5. These results support the concept that CFTR, as well as ClC-5, plays a relevant role in PT cell endocytosis. They also demonstrate that the functional loss of these two chloride transporters is associated with impaired uptake of AG in PT cells, reflected by a decreased renal accumulation of the drug.

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# Increased Renal Clearance of Aminoglycoside Reflects Defective Receptor-mediated Endocytosis in Cystic Fibrosis and Dent's Disease

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**ABSTRACT**

The clinical use of aminoglycoside (AG) antibiotics is limited by their renal toxicity, which is caused by drug accumulation in proximal tubule (PT) cells. Clinical studies reported that renal clearance of AG is enhanced in cystic fibrosis (CF) patients, which might reflect the role of CFTR in PT cell endocytosis. In order to assess the role of chloride transporters on the renal handling of AG, we investigated gentamicin uptake and clearance in mice lacking functional CFTR (*Cftr*<sup>ΔF/ΔF</sup>) or knock-out for the Cl<sup>-</sup>/H<sup>+</sup> exchanger ClC-5 (*Clcn5*<sup>Y/-</sup>). The latter represent a paradigm of PT dysfunction and defective receptor-mediated endocytosis. As compared with controls, *Cftr*<sup>ΔF/ΔF</sup> and *Clcn5*<sup>Y/-</sup> mice showed a 30% to 60% increase in renal gentamicin clearance, respectively. The enhanced clearance was associated with a lower renal accumulation of gentamicin. Studies on primary cultures of *Cftr*<sup>ΔF/ΔF</sup> and *Clcn5*<sup>Y/-</sup> mouse PT cells confirmed the reduction in gentamicin uptake, although colocalization with endosomes and lysosomes was maintained. Quantification of endocytosis in PT cells revealed that gentamicin, similar to albumin, preferentially binds to megalin. The functional loss of ClC-5 or CFTR was reflected by a decrease of the endocytic uptake of gentamicin, with a more pronounced effect in cells lacking ClC-5. These results support the concept that CFTR, as well as ClC-5, plays a relevant role in PT cell endocytosis and also demonstrate that their functional loss is associated with impaired uptake of AG in PT cells, causing an enhanced renal clearance of the drug.

INTRODUCTION

Aminoglycosides (AG) are among the most commonly used antibiotics worldwide. Their activity against a wide range of Gram-negative bacteria combined with a low rate of reversible resistance make AG the drug of choice to treat life-threatening infections. However, nephrotoxicity of AG, which is observed in up to 25% of treated patients, represent a serious adverse event and is a dose limiting factor for their therapeutic use [1,17]. AG are commonly used to treat exacerbations of pulmonary infections (particularly with *Pseudomonas aeruginosa*) in patients with cystic fibrosis (CF). Nephrotoxicity of AG is particularly burdensome in such patients, considering the tenacity of infections and the necessity for repeated treatment courses [24]. A further aspect is that CF patients show enhanced renal clearance for many drugs including AG [13,28]. The molecular basis of such enhanced drug clearance in CF – increase in filtration, decrease in tubular reabsorption or increase in tubular secretion – is debated and is probably drug-specific [25].

Aminoglycosides are non-protein-bound drugs characterized by low tissue penetrance and free glomerular filtration. Around 80% of the administered dose is excreted into the urine within 24h. However, up to 5% of the dose is reabsorbed in the proximal tubule (PT), where the drug accumulates for a long time (half-life >100h), leading to renal damage [22,31]. Aminoglycoside-induced kidney failure is typically non-oliguric, with evidence of PT dysfunction (renal Fanconi syndrome) and occasional toxicity in more distal tubular segments, as reflected by NaCl or magnesium wasting. However, accumulation within the renal cortex, especially in PT cells, is thought to be play a key role in the pathogenesis of AG nephrotoxicity [14].

Experiments in rat models have revealed that accumulation of AG in PT cells is mediated by the multi-ligand receptor, megalin [16]. Megalin and its co-receptor cubilin are abundantly expressed at the apical surface of PT cells, where they mediate the main endocytic pathway for clearance of low-molecular-weight (LMW) proteins that are ultrafiltered by the glomerulus [4]. Rare human disorders caused by loss-of-function mutations in the genes coding for megalin or cubilin, as well as animal models with invalidated megalin or cubilin receptors are invariably associated with urinary loss of LMW ligands [6]. Apart from these receptors, recent studies have revealed that intravesicular chloride transporters may also play an important role in the reabsorption of ultrafiltered ligands [12]. In particular, the vesicular  $\text{Cl}^-/\text{H}^+$  exchanger, CIC-5, is crucial for receptor-mediated endocytosis and megalin/cubilin trafficking in PT cells [5,19,30]. Mutations of the *CLCN5* gene that encodes CIC-5 cause Dent's disease (OMIM #30009), a rare X-linked renal tubulopathy characterized by a generalized dysfunction of the PT associated with LMW proteinuria [8]. More recently, Jouret et al. have demonstrated that loss-of-function mutations in the CF transmembrane conductance regulator (*CFTR*, *ABCC7*) gene induce a moderate but significant PT alteration of LMW protein handling [11]. Indeed, in mouse kidney, CFTR co-distributes with CIC-5 and the vacuolar-ATPase in endosomes located in the apical area of PT cells. Furthermore, lack of CFTR in renal PT cells induces instability of cubilin at the brush border, leading to its accelerated shedding into urine and LMW proteinuria in CF mice and patients [11]. Accordingly, a reduced uptake of AG by PT cells could provide a mechanistic explanation for the enhanced renal clearance of AG observed in CF.

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In order to test this hypothesis and investigate kidney handling of AG, we performed *in vivo* and *in vitro* studies to characterize gentamicin handling by PT cells. We used two clinically relevant animal models, i.e. mice lacking functional CFTR (*Cftr*<sup>ΔF/ΔF</sup>) and those knock-out for ClC-5 (*Clcn5*<sup>Y/-</sup>), a well-established model for Dent's disease and defective PT cell endocytosis.

For Peer Review

## MATERIALS AND METHODS

### Mouse models

Experiments were conducted on age- and gender-matched *Cftr*<sup>+/+</sup> and mutated *Cftr*<sup>ΔF/ΔF</sup> mice (FVB/129 background) [29]; and *Clcn5*<sup>Y/+</sup> and *Clcn5*<sup>Y/-</sup> mice (C57BL6 background) [30]. All mice had free access to appropriate standard diet (Carfil Quality, Oud-Turnhout, Belgium). The tap water of the *Cftr* mice was enriched with Movicol (55.2 g/L; Norgine, Heverlee, Belgium). The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Ethics Committee of the Université catholique de Louvain.

### Clearance studies

Mice were injected with a therapeutic dose (5mg/kg body weight) gentamicin (Schering-Plough, Brussels, Belgium) [9]. Blood was obtained by retro-orbital puncture after 30 min, under anesthesia with Anesketin (Eurovet, Brussels, Belgium) and Rompun (Bayer, Brussels, Belgium). Subsequently mice were kept in metabolic cages for 24h with *ad libitum* access to food and drinking water. Urine was collected and blood was obtained by puncture of the vena cava at the time of killing. Plasma and urine concentrations of gentamicin were determined using an AxSYM System (detection limit: 0.2 µg/mL; Abbott Laboratories, Abbott Park, IL, USA). The renal gentamicin clearance was measured using the formula  $U \times V / P$ , where U is the urine gentamicin concentration, V the total urine volume, and P the plasma gentamycin concentration. Biological parameters in plasma and urine were measured with a Synchron CX5 analyzer (Beckman Coulter,

Fullerton, CA), whereas Clara Cell protein (CC16) concentration, a 16-kD marker of PT dysfunction, was determined in duplicate by latex immunoassay [30].

A kidney was split in two parts, and half was fixed for 6h at 4°C in 4% 0.1 M phosphate buffered (pH 7.4) formaldehyde (Boehringer Ingelheim, Heidelberg, Germany) and the other half was flash-frozen and stored at -80°C. The contralateral kidney was homogenized in 0.25 M sucrose and 3 mM imidazole Buffer (pH 7.4) that contained Complete (Roche), in a Potter-Elvehjem tissue homogenizer (Thomas Scientific, Swedsboro, NJ) [11]. The homogenate was centrifuged at 1000 X g for 15 min at 4°C and the supernatant were analyzed biochemically for gentamicin concentration using the AxSYM System.

Primary cell culture

Primary mouse proximal tubule cells (mPTC) were cultured under sterile conditions from collagenase-digested cortical fragments of kidneys isolated from male *Cln5* and *Cftr* mice as previously described [26,27]. Seven-day-old confluent monolayers, which retain their differentiation and endocytic capacity [20] were used for experiments.

Immunostaining

Kidney samples and mPTC were fixed in 4% formaldehyde before embedding in paraffin as described [11]. After incubation with 10% serum, kidney sections or mPTC were incubated with primary antibodies diluted in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). After washing, sections were incubated with biotinylated secondary anti-IgG antibodies (Vector Laboratories, Brussels, Belgium).



Sections were viewed under a Leica DMR coupled to a Leica DC300 digital camera (Leica, Heerbrug, Switzerland). For immunofluorescence, Alexa-conjugated secondary anti-IgG antibodies were used (Invitrogen, Merelbeke, Belgium). Nuclei were stained with DraQ5™ (Biostatus, UK). Filters were cut from the holder and mounted in Prolong Gold Anti-fade reagent (Invitrogen, Belgium). Sections were analyzed under a Zeiss LSM510Meta Confocal microscope (Zeiss, Belgium).

### Antibodies

Immunostaining analyses were performed using well-characterized rabbit monoclonal antibody against gentamicin (a gift from K. Fujiwara, Sojo University, Japan) [10]; mouse monoclonal antibody against early endosomal antigen-1 (EEA1); and polyclonal antibodies against megalin (a gift from P. Verroust, INSERM, Paris, France).

### Gentamicin Endocytosis

Characterization of the gentamicin uptake was performed in fixed and live mPTC. Confluent monolayers of mPTC, isolated from *Cln5* and *Cftr* mice, were exposed to 5 mg/ml gentamicin for 7 or 45 min at 37°C, fixed in 4% formaldehyde (see above) and subsequently stained with anti-EEA1 and anti-gentamicin. For live-cell imaging, confluent monolayers of mPTC were simultaneously incubated with 1 mg/ml Alexa 488-gentamicin and Lysotracker Red (Invitrogen, Merelbeke, Belgium) for 7 min at 37°C and washed. Fluorescence was chased up to 45min after exposure of the cells to gentamicin. Cells were examined using the Zeiss LSM510Meta confocal microscope.

Quantification of endocytosis was performed as previously described [20]. Confluent mPTC were incubated with Alexa488-conjugated gentamicin or Alexa488-conjugated BSA (both at 0.5 mg/ml) for 15 min at 37°C. After incubation, mPTC were washed 5 times with cold HBSS (4°C) and lysed. The fluorescence in the lysate was measured in a fluorimeter (Perkin Elmer) with excitation at 490±10 nm and emission at 525±10 nm. For metabolic inhibition experiments, mPTC were incubated with 10mM sodium azide or 50mM 2-deoxyglucose (DOG) for 30 min at 37°C before the endocytic assay. For competition experiments, mPTC were simultaneously incubated with 50mM unlabelled albumin or transferrin. Fluorescence was normalized for the amount of protein in the cells, measured using the BCA kit (Pierce, Belgium).

Data are expressed as mean ± SEM. Significance of differences was assessed by unpaired, two-tailed Student's t-test.

## RESULTS

### *Clearance of gentamicin in Cftr and Clcn5 mice*

We first investigated renal function and gentamicin clearance in  $Cftr^{AF/AF}$  and  $Clcn5^{Y/-}$  mice in comparison with their corresponding control littermates. Clearance values and renal accumulation of gentamicin were obtained in mice injected i.p. with gentamicin and kept in metabolic cages for 24h. Renal function as measured by creatinine clearance, BUN and plasma creatinine levels, was similar for CFTR and ClC-5 mice as compared with their wild-type controls (Table 1). However,  $Cftr^{AF/AF}$  mice showed a significant increase in the urinary albumin excretion as well as LMW proteinuria as evidenced by the increased urinary excretion of CC16. The  $Clcn5^{Y/-}$  mice also presented with albuminuria and LMW proteinuria, in a greater extent than that observed in  $Cftr^{AF/AF}$  mice. The  $Clcn5$  knock-out mice also showed polyuria (Table 1).

Analysis of gentamicin handling (Table 2) showed that  $Cftr^{AF/AF}$  mice had a 24% increase in the gentamicin clearance, as reflected by a lower kidney accumulation of gentamicin. The  $Clcn5^{Y/-}$  mice showed a higher (66%) increase in gentamicin clearance as compared to controls, with very low levels of gentamycin in  $Clcn5^{Y/-}$  kidneys. These results show that, even in absence of renal failure, the clearance of gentamicin is significantly increased in mouse models of CF and Dent's disease in parallel with the degree of PT dysfunction in these models.

### *Immunolocalization of gentamicin and megalin in Cftr and Clcn5 kidneys*

To substantiate the clearance data, we performed immunostaining for gentamicin (Fig. 1, panel A) on kidney sections taken from  $Cftr$  and  $Clcn5$  mice 24h post-injection.

Gentamicin was clearly detectable in PT sections of both *Cftr*<sup>+/+</sup> and *Clcn5*<sup>Y/-</sup> kidneys, contrasting with decreased immunoreactivity in *Cftr*<sup>ΔF/ΔF</sup> and *Clcn5*<sup>Y/-</sup> kidneys. A residual, faint signal for gentamicin could be observed in PT profiles from the *Cftr*<sup>ΔF/ΔF</sup> while no residual signal was detected in *Clcn5*<sup>Y/-</sup> mice. Since gentamicin preferentially binds to megalin, we investigated the immunoreactivity for megalin in kidney sections of gentamicin exposed *Cftr* and *Clcn5* mice (Fig. 1, panel B). In comparison with wild-type kidneys, only a faint signal for megalin could be observed at the apical region of PT in *Clcn5*<sup>Y/-</sup> kidneys, whereas megalin expression was almost unchanged in *Cftr*<sup>ΔF/ΔF</sup> kidneys. These *in vivo* data suggest that the enhanced urinary excretion of gentamicin could result from a decreased receptor-mediated uptake in PT cells.

*Uptake of gentamicin in mPTC from Clcn5 and Cftr mice*

We used a well-characterized mPTC culture system to evaluate the subcellular handling of gentamicin and its dependence on CFTR and ClC-5 in PT cells. Primary cultures of mPTC obtained from wild-type (Fig. 2, panels A-D), *Clcn5*<sup>Y/-</sup> (Fig. 2, panels E-H) and *Cftr*<sup>ΔF/ΔF</sup> (Fig. 2, panels I-L) mice were exposed to gentamicin at 37°C for 7 min (early endocytosis) and 45 min (late endocytosis and lysosomal processing). In comparison with wild-type mPTC (panels A-B), the gentamicin uptake after 7 min was strongly reduced in *Clcn5*<sup>Y/-</sup> cells (panels E-F), while a milder reduction was observed in *Cftr*<sup>ΔF/ΔF</sup> mPTC (panels I-J). At 45 min, numerous large gentamicin-positive vesicles could be observed in wild-type mPTC (panels C-D). A smaller number of positive vesicles was detected in *Clcn5*<sup>Y/-</sup> (panels G-H) and *Cftr*<sup>ΔF/ΔF</sup> (panels K-L) mPTC.

We next used mPTC to identify the localization of gentamicin within the endosomal-lysosomal pathway. Monolayers of mPTC were double stained for gentamicin and anti-early endosomal antigen-1 (EEA1) after incubation with gentamicin for 7 min (Fig. 3). In wild-type mPTC, gentamicin colocalized with EEA1, confirming its presence in the early endosomes (Fig. 3, panels A-C). By contrast, almost no gentamicin could be detected in *Clcn5*<sup>Y/-</sup> mPTC (Fig. 3, panel D), whereas a markedly reduced staining was detected in *Cftr*<sup>ΔF/ΔF</sup> mPTC (Fig. 3, panel G). The few vesicles that contained gentamicin stained positive for EEA1, indicating the involvement of early endosomes (Fig. 3, panel I).

A pulse-chase experiment was performed to investigate the progression of gentamicin into the lysosomal compartment of mPTC (Fig. 4). Monolayers of wild-type (Fig. 4, panels A-C), *Clcn5*<sup>Y/-</sup> (Fig. 4, panels D-F) and *Cftr*<sup>ΔF/ΔF</sup> mPTC (Fig. 4, panels G-I) were exposed to Alexa 488-gentamicin and Lyso Tracker Red and gentamicin localization was analyzed after 45 min. In wild-type mPTC, gentamicin colocalized with LysoTracker Red (panel C) confirming intra-lysosomal capture. A reduction of gentamicin uptake was detected in *Clcn5*<sup>Y/-</sup> and *Cftr*<sup>ΔF/ΔF</sup> mPTC (panels D and G, respectively), with occasional colocalization of few gentamicin-positive vesicles with the lysosomal marker (panels F and I). Taken together, these data demonstrate that uptake of gentamicin and its progression along the endosomal-lysosomal pathway are affected by the functional loss of CFTR or ClC-5 in PT cells.

#### *Quantification of gentamicin endocytosis in mPTC from Clcn5 and Cftr mice*

To monitor the effect of CFTR or ClC-5 loss on gentamicin uptake, we incubated mPTC from *Clcn5* and *Cftr* mice for 15 min with similar, non-toxic concentrations of

Alexa488-gentamicin or Alexa488-BSA, taken as a well-established ligand for megalin. The uptake of gentamiycin or albumin was then analyzed (i) under control conditions, (ii) in the presence of competitive inhibitors of megalin (excess of albumin) or cubilin (excess of transferrin), and (iii) in conditions of metabolic inhibition (Fig. 5). In control mPTC, the amount of accumulated gentamicin was similar to that of albumin ( $70 \pm 4$  vs.  $71 \pm 2$  ng/ $\mu$ g protein respectively). Competitive or metabolic inhibition could block gentamicin and albumin uptake by mPTC. As compared to wild-type mPTC, gentamycin and albumin uptake was reduced by ~75% in *Clcn5*<sup>Y/-</sup> mPTC and by ~20% in *Cftr* <sup>$\Delta F/\Delta F$</sup>  mPTC. These results show that functional loss of ClC-5 or CFTR is reflected by a significant loss in the endocytic uptake of gentamicin (and albumin) by PT cells; this effect is much more pronounced in cells lacking ClC-5 than in those lacking CFTR.

## DISCUSSION

In this study, we analyzed the handling of AG by PT cells using *in vivo* and *in vitro* experiments performed on *Cftr*<sup>ΔF/ΔF</sup> and *Clcn5*<sup>Y/-</sup> mice. The increased gentamicin clearance we observed in both strains (+30% in *Cftr*<sup>ΔF/ΔF</sup> vs. +60% in *Clcn5*<sup>Y/-</sup> mice) was associated with a lower accumulation of the drug in the kidney. The reduction in gentamicin uptake was confirmed in mPTC obtained from *Cftr*<sup>ΔF/ΔF</sup> and *Clcn5*<sup>Y/-</sup> mice, with much fewer gentamicin-positive vesicles in the early endosomal and lysosomal compartments. Quantification of endocytosis in mPTC revealed a similar profile of uptake and inhibition for gentamycin and albumin. The endocytic uptake of albumin and gentamicin was shown to be significantly reduced in *Cftr*<sup>ΔF/ΔF</sup> and *Clcn5*<sup>Y/-</sup> mPTC, but this effect was much more pronounced in cells lacking ClC-5 than in those lacking CFTR. These results confirm the role of CFTR in PT cell endocytosis and demonstrate that the functional loss of CFTR, as that of ClC-5, is associated with a decreased efficacy of receptor-mediated uptake in PT cells, causing an enhanced AG clearance.

Among other factors, aggressive antibiotic treatment for lung infections has led to a dramatic increase in life expectancy for patients with CF over the last three decades [24]. Because of their efficacy against *Pseudomonas aeruginosa*, AG antibiotics are particularly useful to treat chronic lung infections in patients suffering from CF [2,21]. In the general population, up to 25% of treated patients will present AG-induced nephrotoxicity, despite accurate follow-up [14]. The renal toxicity of AG is clearly related to their uptake along the PT segments via receptor-mediated endocytosis [22], accumulation in endo-lysosomal vesicles, modification of the membrane phospholipid content, leakage in the cytosol, and subsequent toxicity for mitochondria and

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3 endoplasmic reticulum, activating different pathways leading to cell death [14,23]. It is  
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5 known for a long time that CF patients display an enhanced renal clearance for several  
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7 drugs including AG [13,25,28]. Since protein binding is generally unchanged in CF  
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9 patients and no significant differences in glomerular filtration rate (GFR) have been  
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11 found in most studies [25,28], a possible role for defective endocytic uptake has been  
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13 evoked. This hypothesis has first been substantiated by Jouret et al., who evidenced a  
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15 defect in receptor-mediated endocytosis in CF mouse models and CF patients [11]. These  
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17 studies revealed that CFTR, like ClC-5, is expressed in the endosomes of PT cells [7].  
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19 Mice knock-out for CFTR or harboring the F508del mutation presented with a mild but  
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21 significant albuminuria and LMW-proteinuria, caused by a defective receptor-mediated  
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23 endocytosis. Patients with CF showed a similar defect in LMW protein handling [11].  
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29 To investigate the putative link between defective endocytosis and changes in  
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31 gentamicin handling in CF, we compared the *Cftr*<sup>ΔF/ΔF</sup> mouse vs. the *Clcn5*<sup>Y/-</sup> mouse  
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33 model of Dent's disease. The *Cftr*<sup>ΔF/ΔF</sup> mouse model bears the most common mutation in  
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35 CF (F508del) [29], which results in misfolding and lack of maturation of CFTR that  
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37 cannot reach the apical plasma membrane. Instead, the mutated protein does not escape  
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39 the endoplasmic reticulum quality control and is degraded by the ubiquitin-proteasomal  
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41 pathway [3]. However, the *Cftr*<sup>ΔF/ΔF</sup> tissues show a residual Cl<sup>-</sup> conductance, suggesting  
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43 that the mutant F508del-CFTR is partially processed and reaches the plasma membrane  
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45 in several tissues including the kidney [3,18]. This residual activity may explain, at least  
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47 partially, the less severe PT phenotype in the *Cftr*<sup>ΔF/ΔF</sup> mouse as compared with the  
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49 *Clcn5*<sup>Y/-</sup> mouse which displays a severe trafficking defect of megalin and cubilin resulting  
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51 in a massive endocytic defect and generalized PT dysfunction [5,19,30]. Our data  
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confirm the quantitative difference in LMW proteinuria and extend it to the renal handling of gentamicin. The increased gentamicin clearance in both models could result from a decreased reabsorption as evidenced by a lower kidney gentamicin accumulation as assessed by immunostaining and quantitative AG analyses in kidney extracts.

Previous studies have identified receptor-mediated endocytosis through binding of megalin as the primary route of uptake for polybasic drugs such as AG [16,22]. Our *in vivo* studies show a mild (*Cftr*<sup>ΔF/ΔF</sup>) or dramatic (*Clcn5*<sup>Y/-</sup>) reduction in megalin staining in PT segments, which could explain the enhanced gentamicin clearance and the decreased renal accumulation. For a more comprehensive investigation of gentamicin handling by PT cells, we used primary cultures of mPTC which remain well differentiated and keep essential properties such as receptor-mediated endocytosis [27]. For instance, we recently showed that the endocytic defect of *Clcn5*<sup>Y/-</sup> mice was retained in primary cultured mPTC [20], and that these cells were responding to a transcriptional program of differentiation [26]. Using this system, we demonstrated that the lack of functional CFTR leads to a significant, ~20% decrease in gentamicin and albumin uptake, as compared with a major, ~80% reduction in cells KO for ClC-5. The fact that similar proportions were obtained for gentamicin clearance and kidney accumulation highlights the correspondence between the distinct methodological approaches targeting the endocytic defect *in vivo* and *in vitro*. The sensitivity of gentamicin uptake to metabolic and competitive inhibition (with both excess of albumin and transferrin) identified the energy-demanding receptor-mediated endocytosis pathway using the multiligand receptors megalin and cubilin as the primary uptake route for gentamicin in mPTC. Our investigation focusing on the trafficking of gentamicin by colocalization with endosomal and lysosomal markers

confirmed the AG processing in PT cells. In wild-type mPTC, gentamicin colocalized with EEA1 in the early endosomal pathway after 7 min incubation, whereas most of the gentamicin was localized in lysosomes after 45 min. The gentamicin uptake was reduced in *Cftr*<sup>AF/AF</sup> and *Clcn5*<sup>Y/-</sup> mPTC, but rare colocalization with endosomal and lysosomal markers could be observed. The fact that gentamicin is taken up by receptor-mediated endocytosis and routed to lysosomes opens possibilities to prevent drug accumulation in the PT and thus limiting nephrotoxicity. The search for strategies to prevent the binding of gentamicin to megalin has been initiated [14,15]. Finally, the enhanced excretion observed in the mouse models tested here may suggest that dosage adaptation and drug monitoring is particularly important when using AG in patients suffering from a defective endocytosis and LMW-proteinuria, including CF and Dent's disease.

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For Peer Review

## FIGURE LEGENDS

### **Figure 1: Gentamicin uptake and megalin expression in *Cftr* and *Clcn5* kidneys.**

Panel A. Immunostaining for gentamicin in *Cftr* and *Clcn5* kidneys 24h post-injection of gentamicin (5mg/kg) showing segmental gentamicin staining in the proximal tubules (PT) of both wild-type *Cftr*<sup>+/+</sup> and *Clcn5*<sup>Y/+</sup> kidneys. In contrast, the staining is abolished in the proximal tubules of *Clcn5*<sup>Y/-</sup> kidneys and is weakly detected in *Cftr*<sup>ΔF/ΔF</sup> kidneys.

Panel B. Immunostaining for megalin showing a strong expression at the apical region of the cells lining the proximal tubules in the wild-type *Cftr*<sup>+/+</sup> and *Clcn5*<sup>Y/+</sup> kidneys. In contrast, the staining is almost undetectable in *Clcn5*<sup>Y/-</sup> kidneys and is slightly decreased in *Cftr*<sup>ΔF/ΔF</sup> kidneys. Scale bar: 50μm (panel A), 100 μm (panel B).

### **Figure 2: Gentamicin uptake in proximal tubule cells.**

Primary cultures of proximal tubule cells (mPTC) obtained from *Clcn5*<sup>Y/+</sup> (wild-type, panels A-D), *Clcn5*<sup>Y/-</sup> (panels E-H) and *Cftr*<sup>ΔF/ΔF</sup> (panels I-L) kidneys exposed to 5 mg/ml gentamicin (37°C for 7min or 45min). Staining with anti-gentamicin and labeling with Alexa488 IgG were analyzed by confocal microscopy. Nuclei counterstained with DraQ5<sup>TM</sup> (red).

In comparison with wild-type mPTC (A-B), *Clcn5*<sup>Y/-</sup> cells showed a drastically reduced early (7 min) uptake of gentamicin (E-F), while a milder reduction was observed in *Cftr*<sup>ΔF/ΔF</sup> mPTC (I-J). At 45 min, numerous large gentamicin-positive vesicles positive could be observed in wild-type mPTC (C-D). A smaller number of positive vesicles was detected in *Clcn5*<sup>Y/-</sup> (G-H) and *Cftr*<sup>ΔF/ΔF</sup> (K-L) mPTC. No difference in staining could be

observed between mPTC obtained from wild-type  $Clcn5^{Y/+}$  and  $Cftr^{+/+}$  kidneys and therefore a representative image of wild-type mPTC is shown. Scale bar: 10µm.

**Figure 3: Early localization of gentamicin in proximal tubule endosomes.**

Primary cultures of  $Clcn5^{Y/+}$  (wild-type; panels A-C),  $Clcn5^{Y/-}$  (panels D-F) and  $Cftr^{AF/AF}$  (panels G-I) mPTC exposed to 5 mg/ml gentamicin (7 min at 37°C), fixed and stained with anti-gentamicin (Alexa488; A,D&G) and anti-EEA1 (Alexa568; B,E&H). Nuclei counterstained with DraQ5<sup>TM</sup> (blue). Gentamicin colocalizes with EEA1 in multiple vesicles in control mPTC, contrasting with a lower density of vesicles observed in the  $Clcn5^{Y/-}$  and  $Cftr^{AF/AF}$  cells (Merge panels, yellow spots). Scale bar: 10µm.

**Figure 4: Late localization of gentamicin in proximal tubule lysosomes.**

Primary cultures of  $Clcn5^{Y/+}$  (wild-type; panels A-C),  $Clcn5^{Y/-}$  (panels D-F) and  $Cftr^{AF/AF}$  (panels G-I) mPTC exposed to 1 mg/ml Alexa488-gentamicin (green; A,D&G) and LysoTracker Red (red; B,E&H) for 7 min at 37°C and followed up for 45min using live-cell imaging. Gentamicin colocalizes with LysoTracker after 45min (yellow spots, merge panels), but a much lower density of positive vesicles observed in  $Clcn5^{Y/-}$  and  $Cftr^{AF/AF}$  cells. Scale bar: 5µm.

**Figure 5: Quantification of gentamicin uptake in mPTC.**

Primary cultures of wild-type (Wt) mPTC (open bars),  $Clcn5^{Y/-}$  (striped bars) and  $Cftr^{AF/AF}$  (dashed bars) mPTC exposed to 0.5 mg/ml Alexa488-gentamicin (white bars) or 0.5mg/ml Alexa488-BSA (grey bars) for 15min at 37°C under control conditions or in



the presence of excessive amount of competitive inhibitors albumin (+Alb) or transferrin (+Tf) or after metabolic inhibition (MI). The uptake of albumin and gentamicin is decreased in a variable extent in the *Clcn5<sup>Y/-</sup>* vs. the *Cftr<sup>ΔF/ΔF</sup>* mPTC. The residual uptake after competitive inhibition with an excess of ligands or with metabolic inhibition is shown for comparison.

Data are mean ± SEM of 6 experiments (n=6). Statistical differences are indicated with \*p<0.001 and \*\*p<0.01 vs. Wt mPTC.

Table 1. Clinical and biochemical parameters in *Cftr* and *Clcn5* mice

	<i>Cftr</i> <sup>+/+</sup>	<i>Cftr</i> <sup>Δ/Δ</sup>	<i>Clcn5</i> <sup>Y/+</sup>	<i>Clcn5</i> <sup>Y/-</sup>
Body weight (g)	26.2 ± 1.3	25.2 ± 0.9	24.6 ± 0.4	29.1 ± 0.4 <sup>b</sup>
BUN (mg/dL)	25 ± 0.5	27 ± 3.3	27 ± 2.8	25 ± 1.7
Plasma creatinine (mg/dL)	0.072 ± 0.02	0.076 ± 0.02	0.086 ± 0.02	0.08 ± 0.01
Diuresis (μL/24h)	708 ± 87	704 ± 123	996 ± 176	2317 ± 447 <sup>b</sup>
Creatinine clearance (μL/min.g BW)	37 ± 10	40 ± 7	17 ± 4	23 ± 3
Urinary albumin (μg/g creat)	1893 ± 1385	10,869 ± 3513 <sup>a</sup>	798 ± 761	58,608 ± 3041 <sup>c</sup>
Urinary CC16 (μg/g creat)	42 ± 23	132 ± 20 <sup>a</sup>	1.7 ± 0.9	37,665 ± 10,877 <sup>d</sup>

<sup>a</sup>p<0.05 vs. *Cftr*<sup>+/+</sup>; <sup>b</sup>p<0.05 vs. *Clcn5*<sup>Y/+</sup>; <sup>c</sup>p<0.0001 vs. *Clcn5*<sup>Y/+</sup>; <sup>d</sup>p<0.01 vs. *Clcn5*<sup>Y/+</sup>

BUN, Blood urea nitrogen; there were 5 pairs of age- and gender-matched mice per genotype.

**Table 2. Gentamicin handling by *Cftr* and *Clcn5* mice**

	<i>Cftr</i> <sup>+/+</sup>	<i>Cftr</i> <sup>ΔΔ</sup>	<i>Clcn5</i> <sup>Y/+</sup>	<i>Clcn5</i> <sup>Y/-</sup>
<b>Plasma gentamicin (μg/mL)</b>	7.2 ± 0.5	7.9 ± 1.2	6.7 ± 0.6	8.8 ± 0.7
<b>Gentamicin clearance (mL/min)</b>	5.2 ± 0.4	6.6 ± 0.7 <sup>a</sup>	5.6 ± 0.5	9.3 ± 1.0 <sup>b</sup>
<b>Kidney gentamicin (μg/mL)</b>	0.89 ± 0.1	0.75 ± 0.1 <sup>a</sup>	0.66 ± 0.1	0.1 ± 0.05 <sup>c</sup>

<sup>a</sup> p<0.05 vs. *Cftr*<sup>+/+</sup>; <sup>b</sup> p<0.01 vs. *Clcn5*<sup>Y/+</sup>; <sup>c</sup> p<0.0001 vs. *Clcn5*<sup>Y/+</sup>

There were 5 pairs of age- and gender-matched mice per genotype.

Figure 1 – Panel A

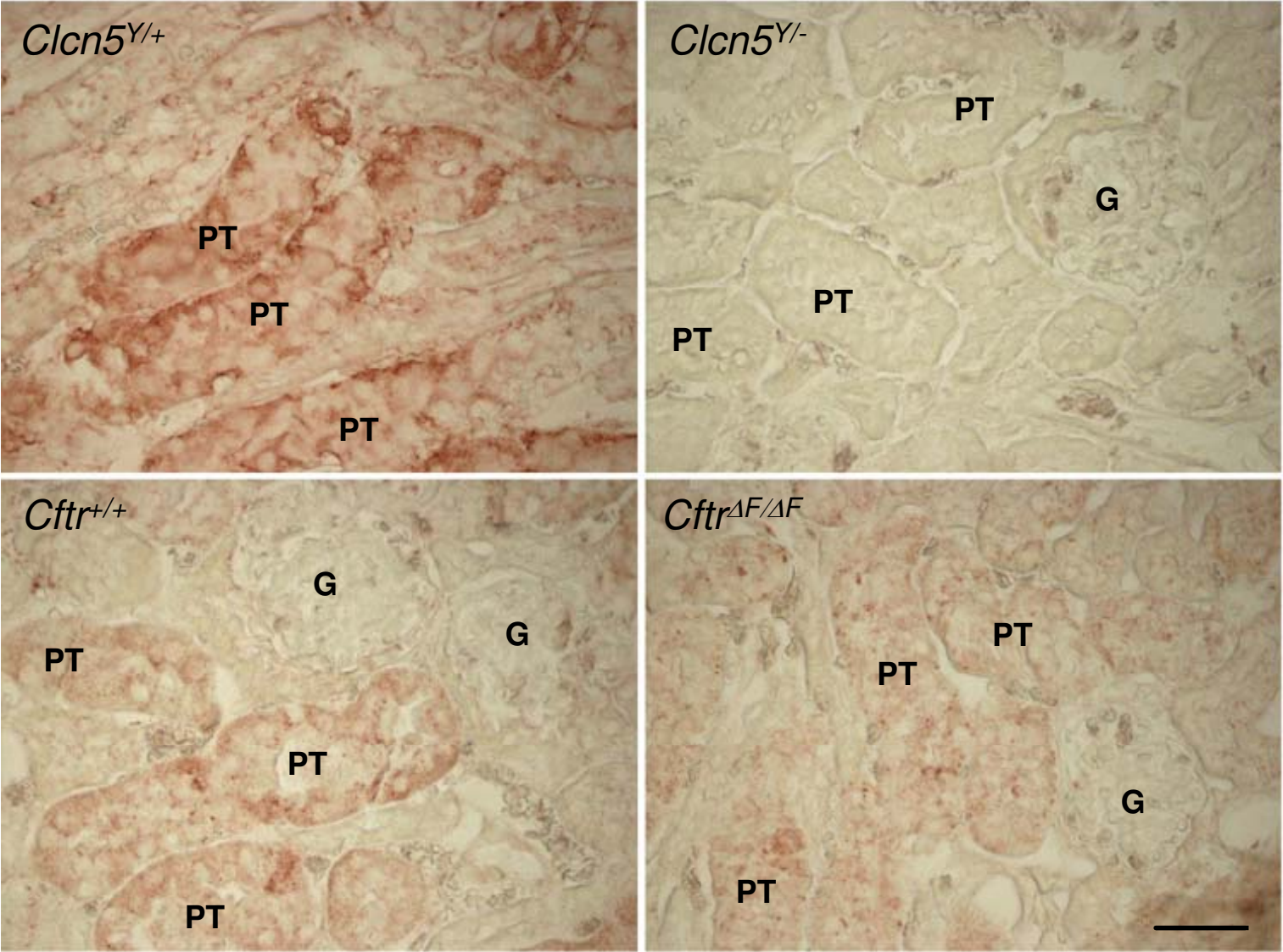




Figure 1 – Panel B

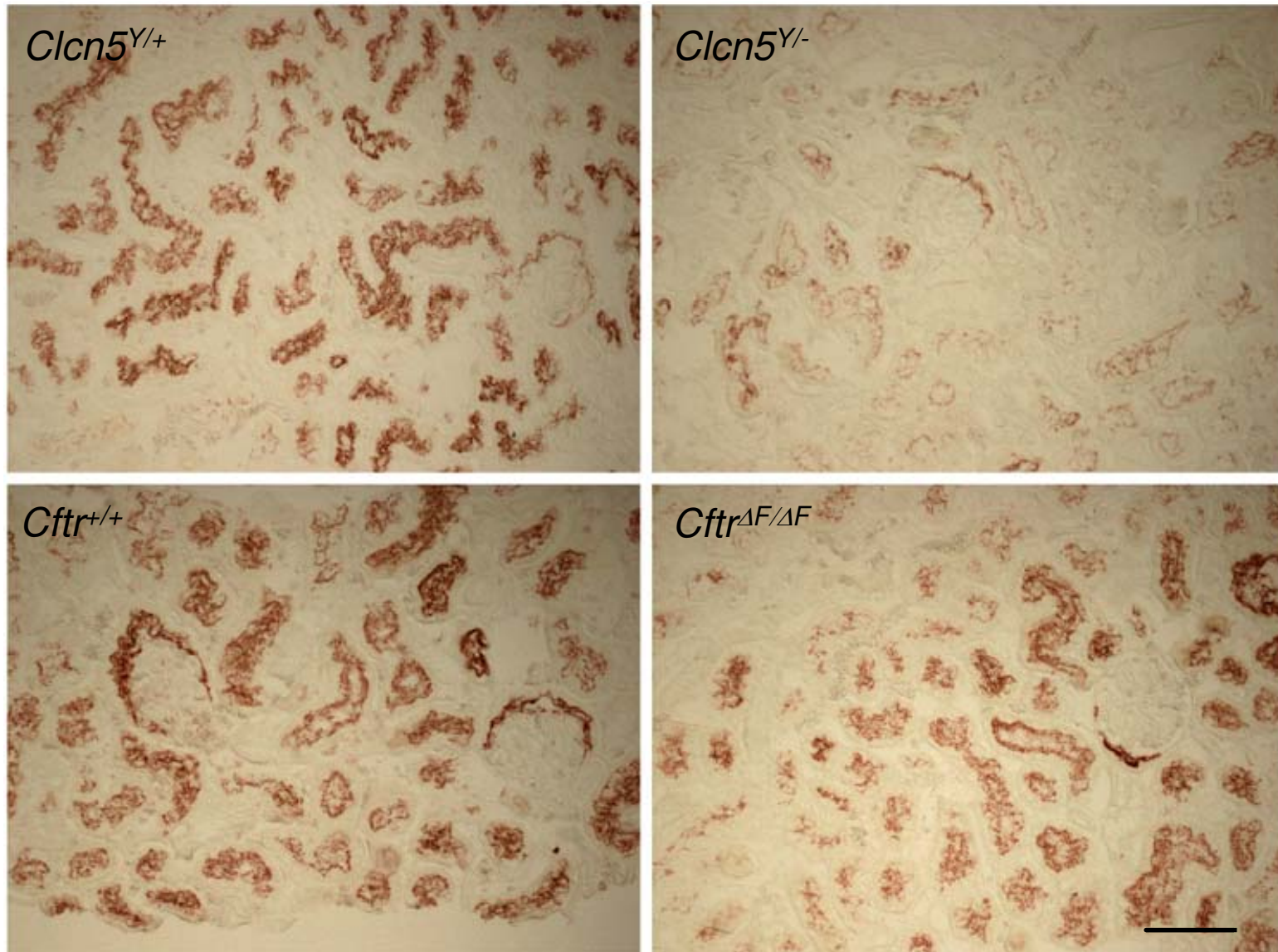


Figure 2

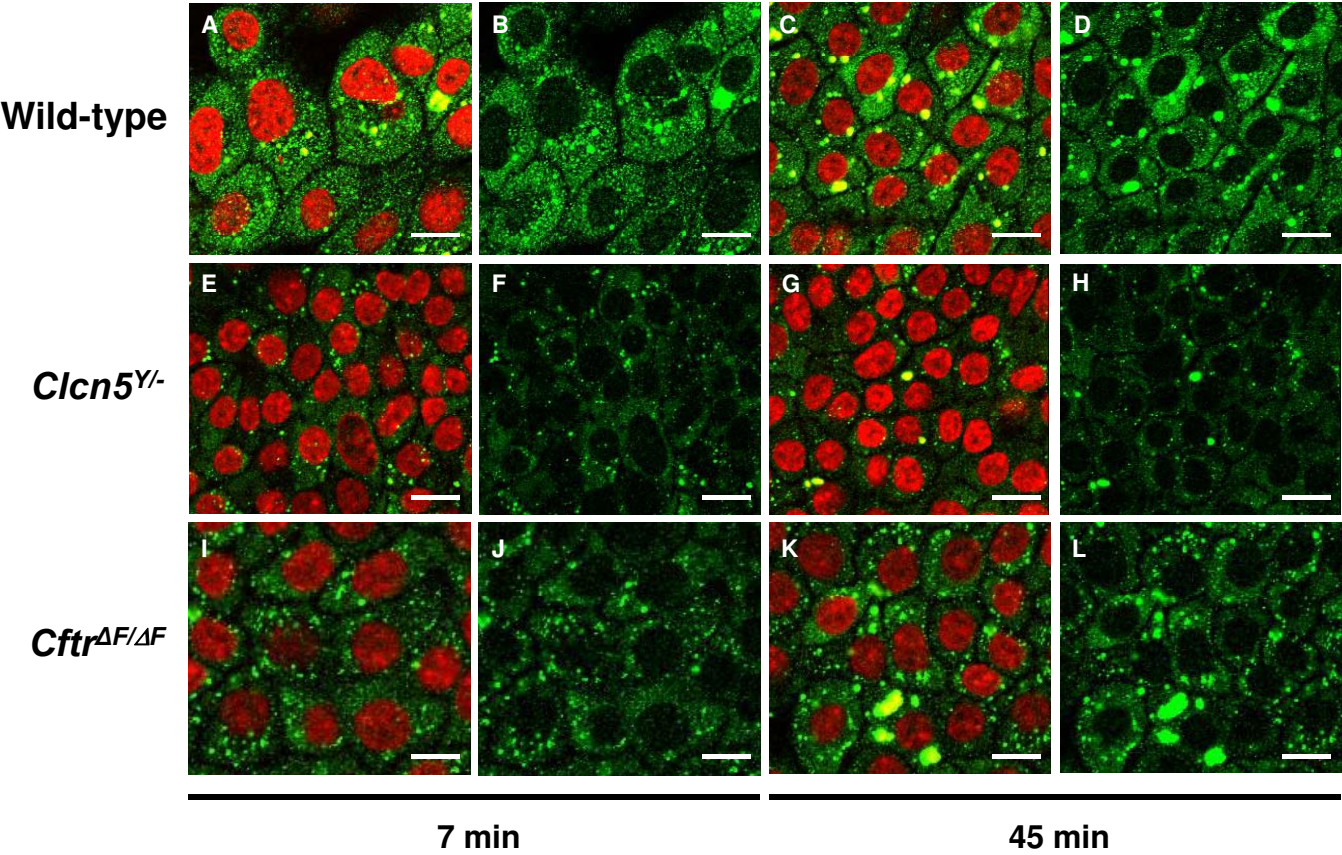




Figure 3

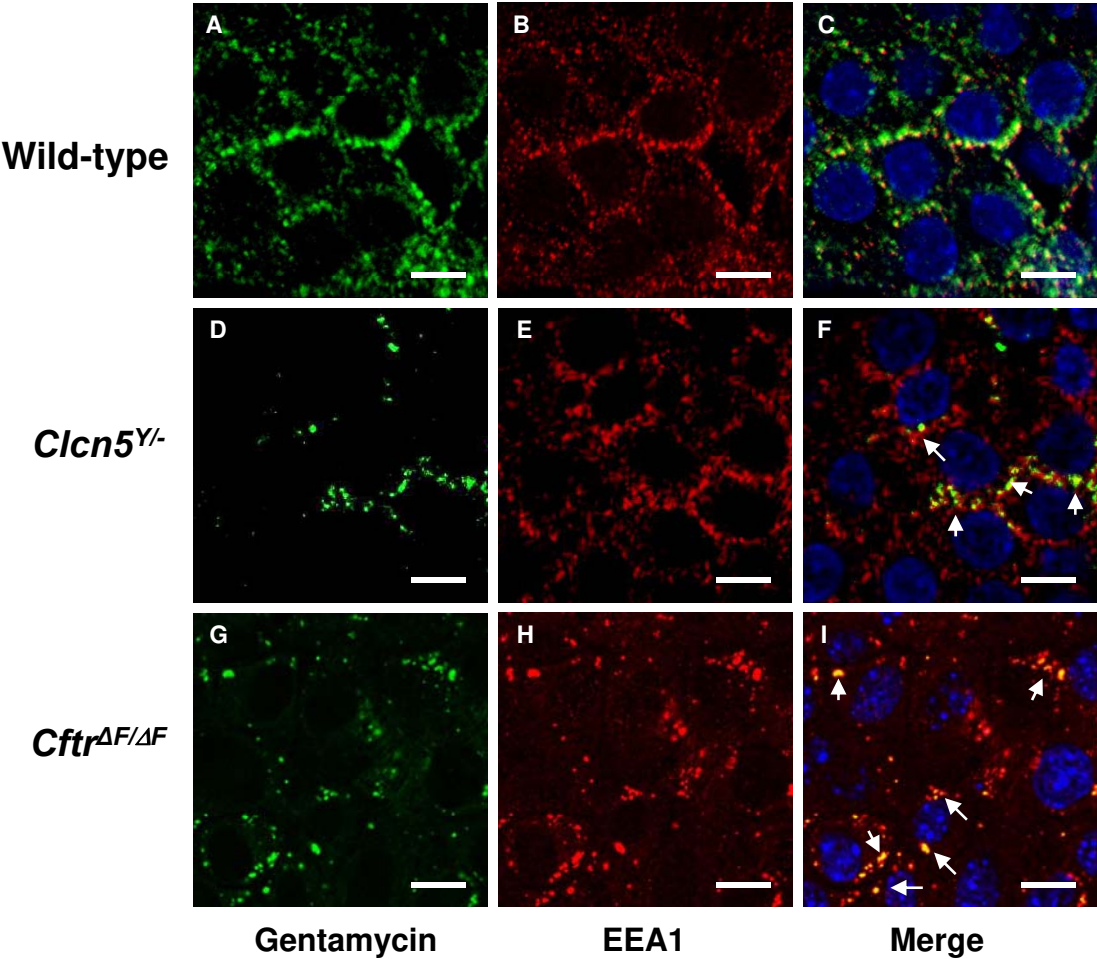


Figure 4

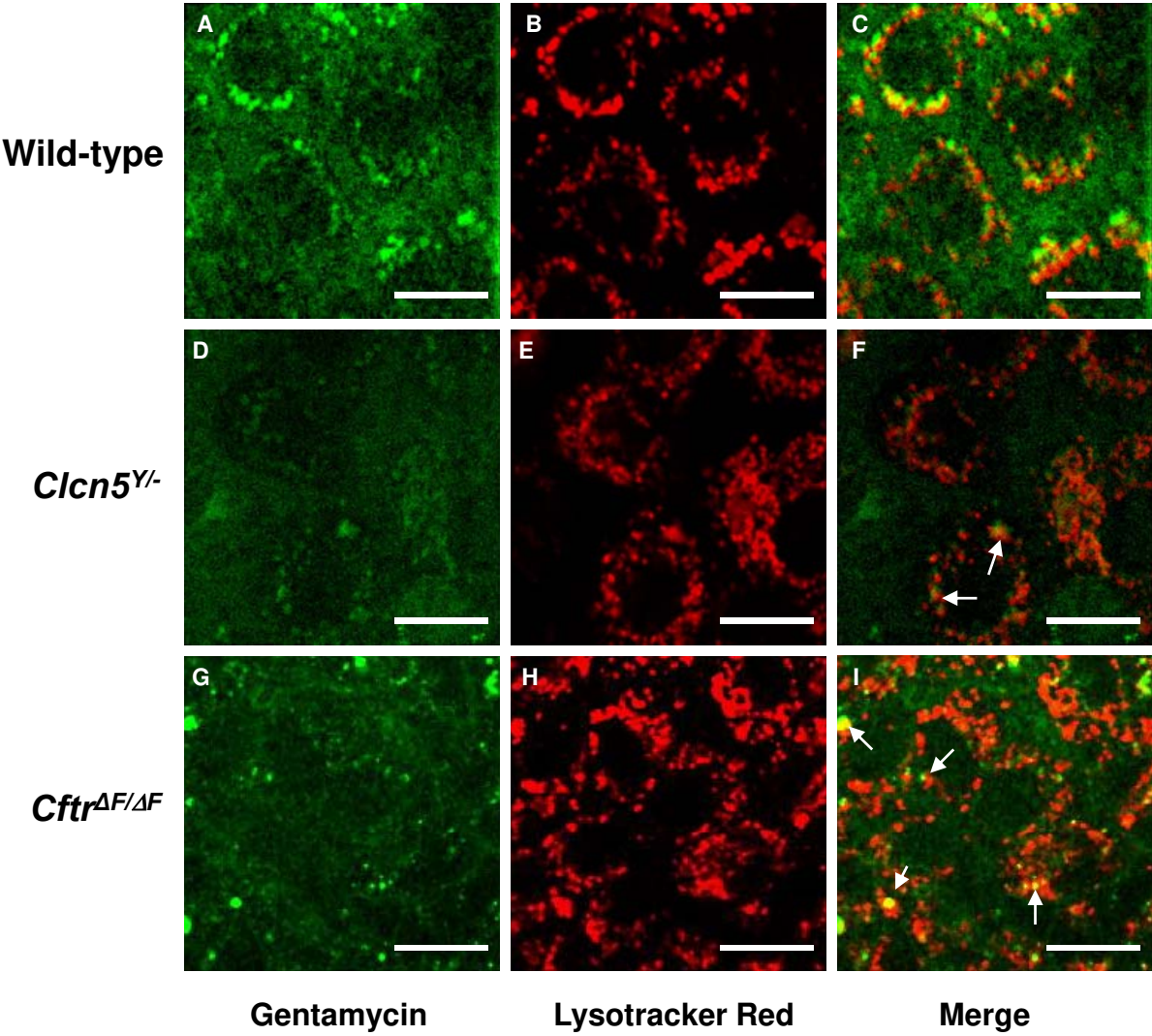




Figure 5

